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Impaired On/Off Regulation of TNF Biosynthesis in Mice Lacking TNF AU-Rich Elements: Implications for Joint and Gut-Associated Immunopathologies

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Summary

We addressed the impact of deleting TNF AU-rich elements (ARE) from the mouse genome on the regulation of TNF biosynthesis and the physiology of the host. Absence of the ARE affected mechanisms responsible for *TNF* mRNA destabilization and translational repression in hemopoietic and stromal cells. In stimulated conditions, TNF ARE were required both for the alleviation and reinforcement of message destabilization and translational silencing. Moreover, the mutant mRNA was no longer responsive to translational modulation by the p38 and JNK kinases, demonstrating that TNF ARE are targets for these signals. Development of two specific pathologies in mutant mice, i.e., chronic inflammatory arthritis and Crohn's-like inflammatory bowel disease, suggests that defective function of ARE may be etiopathogenic for the development of analogous human pathologies.

Introduction

Mechanisms regulating mRNA stability and translation are driven by specific elements carried on mRNA sequences. One major class of these regulatory sequences consists of adenosine-uracil multimers with a characteristic AUUUA pentanucleotide and as such are referred to as AU-rich elements (ARE). These ARE have been mapped in the 3′-untranslated region (3′-UTR) of transcripts encoding cytokines, oncoproteins, and growth and transcription factors (Caput et al., 1986) For some mRNAs, including those encoding *IL-2*, c-*fos*, and *GM-CSF*, the function of the ARE has been strongly associated with the regulation of mRNA stability (Chen and Shyu, 1995; Chen et al., 1998). In addition, a paradigm for translational regulation by the ARE has been established for a number of mRNAs (Kruys et al., 1989).

Tumor necrosis factor (TNF) plays a central role in various immune and inflammatory phenomena (Vassalli, 1992; Pasparakis et al., 1996a). TNF exerts its bioactivity either as a transmembrane molecule or as a soluble protein (Perez et al., 1990; Grell et al., 1995; Alexopoulou et al., 1997) by transducing differential signals ranging from cellular activation and proliferation to cytotoxicity and apoptosis through two distinct TNF receptors, p55 (TNFRI) and p75 (TNFRII) (Vandenabeele et al., 1995). Strong TNF producers include mainly cells of the myeloid lineage such as monocytes and macrophages. In addition, a large variety of other cell types, both hemopoietic and stromal, have been observed to produce TNF, including a number of carcinoma cell lines (for review see Vassalli, 1992).

Considering the pleiotropism of TNF actions, it is not surprising that its biosynthesis is under the control of multiple and complex regulatory mechanisms. TNF transcription is under the control of several factors such as NF-κB and NF-AT (Collart et al., 1990; Goldfeld et al., 1991). Moreover, ample evidence has indicated additional translational controls residing at the 3′-UTR of *TNF* mRNA. Experiments using reporter constructs transfected into cultured cell lines have implicated the 3′-UTR region of *TNF* mRNA in the translationally repressed state in which TNF transcripts are found in nonstimulated conditions (Han et al., 1990). This type of blockade was suggested to be permanent in stromal cell types (Kruys et al., 1993) but readily reversible upon appropriate stimulation in tissues of hemopoietic origin (Beutler et al., 1986; Han et al., 1990). Although evidence on proximal factors regulating TNF biosynthesis is scarce, translational activation of *TNF* mRNA in hemopoietic tissues is shown to occur via the action of stress-activated protein kinases (SAPK) (Lee et al., 1994; Swantek et al., 1997). However, the specific target sequences on *TNF* mRNA accepting such modulatory signals have not yet been defined.

Alterations in the patterns of TNF production have been associated with many inflammatory and autoimmune pathological processes (Vassalli, 1992; van Deventer, 1997). More specifically, the dominant role of TNF in the pathogenesis of rheumatoid arthritis (RA) and idiopathic inflammatory bowel diseases (IBD), of currently unknown etiology, has been demonstrated by recent successful clinical trials using anti-TNF antibodies (Elliott et al., 1994; Targan et al., 1997). However, little is known about the molecular mechanisms that may result in aberrant TNF expression in either RA or IBD. Evidence for a potential role of the 3′-UTR of TNF in arthritis comes from studies using mice carrying 3′-UTR-modified human *TNF* transgenes (Keffer et al., 1991). Furthermore, in spontaneous murine models of autoimmunity such as in the NZB/W mice, reduced levels of TNF, which appear to be pathogenic, are associated with mutations in TNF ARE sequences (Jacob et al., 1996). It is therefore conceivable that interference at the level of ARE functioning may be etiopathogenic in vivo. In the present study, we have directly assessed the in vivo impact of the deletion of *TNF* ARE sequences on both the regulation of TNF biosynthesis and the physiology of the host.

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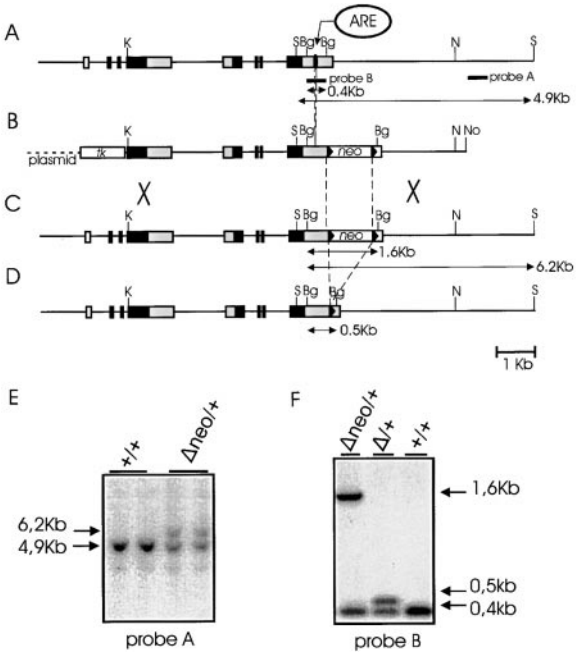

Figure 1. Generation of *TNF*$^{\Delta ARE}$ Mice

(A) Structure of the *TNF/LT*α locus on mouse chromosome 17. (B) The *TNF*$^{\Delta ARE}$ targeting vector and (C) the predicted *TNF*$^{\Delta AREneo}$ mutated locus in embryonic stem cells. Filled boxes represent exons, and gray-shaded boxes represent UTRs. The position of TNF AU-rich elements (ARE) is indicated (dark circle); open boxes denote the selection marker genes. Filled arrowheads indicate the position of the loxP sequences. The location of the external and the internal 3′ probes ([A] and [B], respectively) are also shown. Restriction enzyme sites are as follows: Bg, BglII; Bs, BstEII; S, SacI; K, KpnI; N; NdeI; No, Not. (D) Predicted structure of the mutated *TNF*$^{\Delta ARE}$ locus following Cre-mediated excision of the *neo* marker gene via site-specific recombination on the flanking loxP sequences. (E) Southern blot analysis of SacI-digested tail DNA from *TNF*$^{\Delta AREneo}$ heterozygous F1 mice probed with a 3′ 0.6 kb AccI probe. The positions of the mutant 6.2 kb and wild-type 4.9 kb fragments are indicated. (F) Southern blot analysis of BglII-digested tail DNA from *TNF*$^{\Delta ARE}$ heterozygous F1 mice following Cre-mediated excision of the *neo* marker, detected with a 3′ 0.4 kb BglII probe. The *neo*$^{+}$ (1.6 kb), *neo*$^{-}$ (0.5 kb), and the wild-type (0.4 kb) fragments are indicated.

Results

Generation of *TNF*$^{\Delta ARE}$ Mice

A 69 bp deletion encompassing the TNF ARE was targeted in embryonic stem cells together with the neomycin resistance marker (*neo*) flanked by loxP sequences (Figures 1A–1C). Transient expression of Cre recombinase in fertilized oocytes from these mice resulted in the excision of the *neo* gene and the generation of *TNF*$^{\Delta ARE}$ mouse lines (Figure 1D). Homozygous *TNF*$^{\Delta ARE/\Delta ARE}$ mice displayed early severe pathological manifestations and exhibited a reduced life span (see below). To circumvent this problem, the effects of the *TNF*$^{\Delta ARE}$ mutation on TNF biosynthesis were studied in compound hemizygous *TNF*$^{\Delta ARE/-}$ animals generated by crossing *TNF*$^{\Delta ARE/+}$ to *TNF*$^{-/-}$ mice (Pasparakis et al., 1996b). The use of the hemizygous *TNF*$^{\Delta ARE/-}$ mice in these experiments allowed the investigation of the regulatory role of the ARE element in a monoallelic system devoid of interference by a wild-type *TNF* allele on TNF production.

Chronic Overproduction of TNF in *TNF*$^{\Delta ARE}$ Mice and Increased Sensitivity to Endotoxemia

Examination of *TNF*$^{\Delta ARE}$ sera for murine TNF using a specific ELISA revealed that circulating TNF protein was present in significant quantities in all mutant mice tested. Homozygous *TNF*$^{\Delta ARE/\Delta ARE}$ mice produce as much as 822 ($\pm$ 447) pg/ml of murine TNF at 6 weeks of age, while in heterozygous *TNF*$^{\Delta ARE/+}$ or *TNF*$^{\Delta ARE/-}$ mice values varied between 90 and 430 pg/ml. Normal *TNF*$^{+/+}$ or *TNF*$^{+/-}$ control mice did not show detectable levels of circulating TNF. Serum TNF levels in disease-free *TNF*$^{\Delta ARE/\Delta ARE}$/*TNFRI*$^{-/-}$ mice were at the range of 300 $\pm$ 160 pg/ml. Furthermore, circulating TNF protein levels in the sera of both *TNF*$^{\Delta ARE/-}$ and *TNF*$^{+/-}$ mice were rapidly increased 90 min following LPS administration, reaching three times higher values in the *TNF*$^{\Delta ARE/-}$ mice (3.4 $\pm$ 1.07 ng/ml in *TNF*$^{+/-}$ versus 11.53 $\pm$ 5.4 ng/ml in *TNF*$^{\Delta ARE/-}$ mice). These data demonstrate an overall suppressive role of the ARE on TNF production, resulting in the increased constitutive and inducible production of TNF in the animal. To examine the effect of TNF overproduction on the acute lethal effects of bacterial endotoxin, we compared lethality in *TNF*$^{\Delta ARE/-}$ and *TNF*$^{+/-}$ mice following challenge with varying doses of LPS. *TNF*$^{\Delta ARE/-}$ mice showed increased sensitivity to LPS and a 50% mortality rate at a dose of 100 μg/25 g mouse (5 dead/group of 10 *TNF*$^{\Delta ARE/-}$ versus 0/10 *TNF*$^{+/-}$ mice). In contrast, almost all *TNF*$^{+/-}$ mice survived up to a dose of 400 μg/25 g mouse (1/9 *TNF*$^{+/-}$ versus 7/9 *TNF*$^{\Delta ARE/-}$). These results show that in the absence of the ARE, acute and/or chronic overproduction of TNF in vivo sensitizes the animals to the lethal effects of LPS.

ARE Modulate the Stability of *TNF* mRNA

Macrophages are the most prominent source of TNF production in vivo. We have thus chosen this specific cell type to address the involvement of the ARE in the regulation of TNF biosynthesis. Thioglycollate-elicited peritoneal macrophages (TEPM) and bone marrow–derived macrophages (BMDM) were selected for our analyses. These macrophage populations were isolated from both the original *TNFRI*$^{+/+}$ and disease-free *TNFRI*$^{-/-}$ background (see below) to eliminate the possibility that alterations in TNF biosynthesis in the mutant cells may result from chronic inflammation occurring at the in vivo sites of origin.

At the protein level, both TEPM and BMDM from *TNF*$^{\Delta ARE}$ mice spontaneously produce readily detectable quantities of TNF protein compared to those derived from *TNF*$^{+}$ cultures, which were consistently lower than the detection limit of our ELISA (40 pg/ml) (Table 1). In addition, a 3- to 5-fold increase in TNF protein production, measured 9 hr after LPS stimulation, was detected in macrophage supernatants derived from *TNF*$^{\Delta ARE}$ compared to *TNF*$^{+}$ mice. Therefore, in the absence of an intact ARE, TNF protein production by macrophages remains responsive to LPS stimulation. Moreover, the absence of ARE has an overall enhancing effect on TNF production by macrophages.

To assess the level(s) at which regulation of TNF biosynthesis is affected by the ARE mutation, we compared kinetics of mRNA production in *TNF*$^{\Delta ARE/-}$ *and TNF*$^{+/-}$ TEPM (Figure 2). Using Northern analysis, *TNF*$^{+}$ mRNA

Table 1. Ex Vivo Spontaneous and Induced TNF Protein Production by $TNF^{\Delta ARE}$ Hemopoietic Cells

	$TNF^{+/-}$[a]	$TNF^{\Delta ARE/-}$	$TNF^{+/+}/TNFRI^{-/-}$	$TNF^{\Delta ARE/\Delta ARE}/TNFRI^{-/-}$
TEPM[b]				
Nonstimulated	<0.04[e]	0.30 ± 0.15	<0.04	0.23 ± 0.08
LPS	7.87 ± 2.70	25.04 ± 8.86	11.56 ± 4.55	41.91 ± 7.52
BMDM[c]				
Nonstimulated	<0.04	0.09 ± 0.06	<0.04	0.242 ± 0.12
LPS	7.62 ± 2.64	27.37 ± 2.85	10.10 ± 1.86	51.09 ± 15.76
T cells[d]				
Nonstimulated	0.76 ± 0.09	1.44 ± 0.27	0.56 ± 0.4	1.57 ± 0.5
Anti-CD3	3.37 ± 0.9	10.73 ± 2.98	5.09 ± 1.08	16.75 ± 3.69

Macrophages (5×10^5 adherent cells) and T lymphocytes (5×10^6 cells/ml) were cultured in the presence or absence of LPS (1 μg/ml) or anti-CD3 (10 μg/ml) in medium for 24 hr, and culture supernatants were collected for TNF protein detection using ELISA.
[a] Values (means ± SD) in ng/ml from one of three representative experiments with eight mice per group.
[b] Thioglycollate-elicited peritoneal macrophages.
[c] Bone marrow–derived macrophages.
[d] Nylon wool–enriched splenic T cells.
[e] Detection limit.

could be detected at low quantities in resting macrophages; its levels were rapidly increased 90-fold at its peak at 1 hr after LPS induction and quickly returned to baseline between 3 and 6 hr (Figure 2A). In contrast, $TNF^{\Delta ARE}$ mRNA showed an approximately 2.7-fold increase in resting macrophages, reached a 148-fold induction at its peak at 3 hr after LPS induction, and slowly returned to baseline well past 9 hr. The corresponding transcriptional rates at 0, 1, 3, and 6 hr after LPS stimulation were similar for both $TNF^{\Delta ARE}$ and TNF^{+} alleles (Figure 2B). At 1 hr after LPS stimulation, the 5-fold increase in the rate of wild-type *TNF* gene transcription correlated with a 90-fold increase in the steady-state *TNF* mRNA levels. Notably, in the absence of the ARE, a similar 5-fold increase in transcription resulted in a 46-fold increase in steady-state $TNF^{\Delta ARE}$ mRNA levels, indicating a much reduced yet existent posttranscriptional regulation. We further examined whether a proposed mechanism of induced splicing of pre-mRNA (Yang et al., 1998) could explain the difference in accumulation of $TNF^{\Delta ARE}$ mRNA in macrophages. Induced accumulation (within 15 min of LPS stimulation) and rapid disappearance (by 1 hr after LPS stimulation) of both TNF^{+} and $TNF^{\Delta ARE}$ pre-mRNA could be observed (Figure 2D), suggesting that pre-mRNA splicing mechanisms are not affected by the absence of the ARE in macrophages. Taken together, these results indicate that in the absence of stimulation, ARE act as determinants of *TNF* mRNA instability. However, following LPS stimulation, the ARE mediate posttranscriptional enhancement of *TNF* mRNA accumulation. Calculation of the decay rates for both the wild-type and mutant *TNF* mRNAs following their peak accumulation, at 1 and 3 hr, respectively, revealed a 4-fold increased half-life for the mutant *TNF* mRNA (TNF^{+} mRNA $t_{1/2} = 88$ min versus $TNF^{\Delta ARE}$ mRNA $t_{1/2} = 353$ min) (Figure 2C). Therefore, following peak accumulation of *TNF* mRNA, ARE-mediated negative regulatory mechanisms reestablish default *TNF* mRNA instability.

Similar to what was found in macrophages, $TNF^{\Delta ARE/-}$ spleen-derived, nylon wool–enriched T lymphocytes produce three times more TNF protein (Table 1) and display prolonged mRNA accumulation profiles (data not shown) relative to $TNF^{+/-}$ cultures upon incubation with an agonistic anti-CD3 antibody (Table 1). Increased TNF protein production was also observed in splenic B220-panned lymphocytes in the presence or absence of mitogenic stimulation (data not shown). These results show that in addition to the macrophages, TNF production in other hemopoietically derived cells is equally affected by the absence of the ARE.

The ARE Is a Target for the Regulation of TNF Biosynthesis by the p38/SAPK and JNK/SAPK Pathways

LPS signal transduction in monocytes and macrophages has been shown to involve activation of the mitogen-activated protein kinases (MAPK), p38, p42/44 (ERK), and p54 stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (Lee et al., 1994; Hambleton et al., 1995; Swantek et al., 1997). The observation that selective inhibition of the p38/SAPK with specific imidazole compounds prevents translation of *TNF* mRNA in macrophage cell lines (Prichett et al., 1995) prompted us to investigate the possibility of a direct interaction of this pathway with TNF ARE. Treatment of $TNF^{+/-}$ TEPM for 9 hr with LPS plus the p38/SAPK inhibitor SB203580 resulted in a marked dose-dependent inhibition of LPS-induced TNF protein secretion (Figure 3A), whereas no change in the levels of TNF^{+} mRNA were detected 3 hr after LPS + SB203580 (Figure 3B). Interestingly, similar treatment of $TNF^{\Delta ARE/-}$ macrophages had no effect on either TNF secretion or mRNA accumulation, thereby demonstrating a requirement for the ARE region in the p38/SAPK-mediated activation of *TNF* translation.

Recent studies have shown that dexamethasone (Dex) inhibits *TNF* translation via selective blocking of the JNK/SAPK pathway (Swantek et al., 1997). Significantly reduced levels of TNF protein were measured from LPS-induced $TNF^{+/-}$ TEPM in the presence of Dex; this inhibition occurred in a dose-dependent manner and reached ≥90% inhibition at 10 μM (Figure 3C). At the same dose, Dex was found to also reduce the levels of steady-state TNF^{+} mRNA by 90% (Figure 3D), confirming previous studies reporting the inhibitory effect of Dex on TNF transcription (Beutler et al., 1986). Treatment of $TNF^{\Delta ARE/-}$ TEPM with Dex resulted in a similar reduction in mRNA values. However, even at a maximal

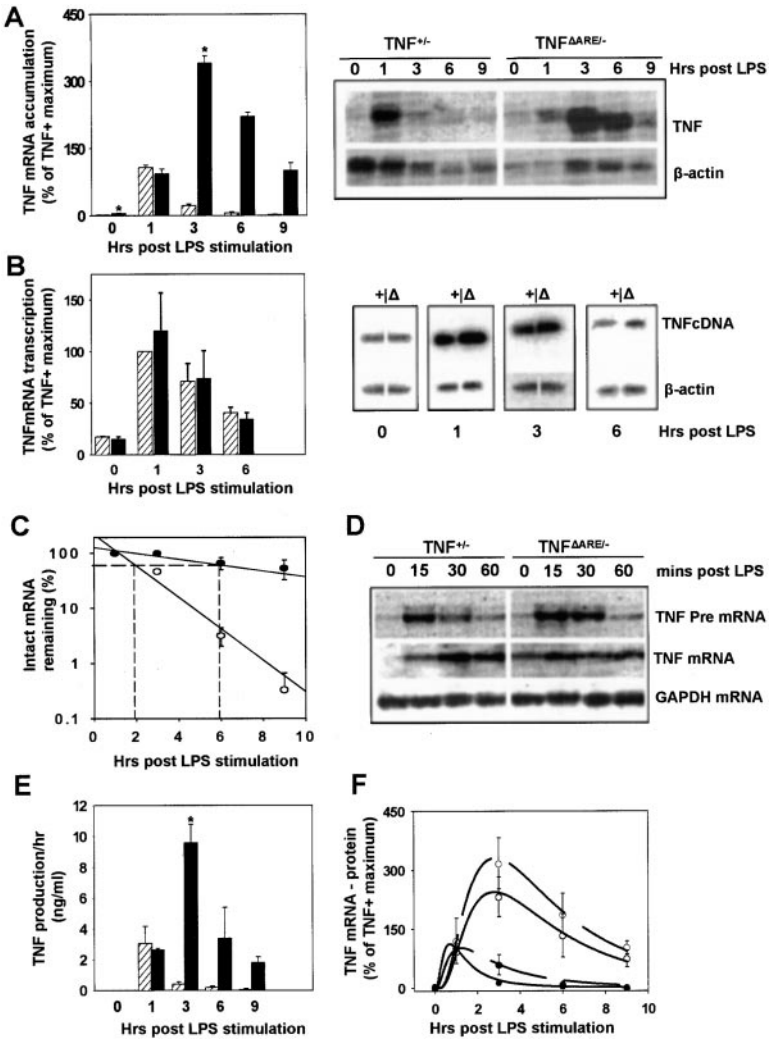

Figure 2. Effect of *TNF*$^{\Delta ARE}$ Mutation on TNF Biosynthesis in Macrophages

(A) Detection of steady-state *TNF* mRNA in *TNF*$^{\Delta ARE/-}$ (closed bars) and *TNF*$^{+/-}$ (hatched bars) macrophages. Total RNA (5–10 μg) from exudate peritoneal macrophage preparations was analyzed on Northern blots. RNA was extracted from adherent cells before (0) or at various time points after stimulation with 1 μg/ml LPS. The bar graph shows the results (mean ± SD) from four independent experiments following scanning quantitation. Data have been normalized according to β-actin values.

(B) Analysis of TNF transcription rate in *TNF*$^{\Delta ARE/\Delta ARE}$/*TNFRI*$^{-/-}$ (closed bars) and *TNFRI*$^{-/-}$ control (hatched bars) macrophages. Nuclei run-on experiment with labeled RNAs obtained from nuclei prepared from exudate peritoneal macrophages treated as above in the presence or absence of LPS. RNAs were hybridized with DNA fragments corresponding to m*TNF* cDNA (1.6 kb) and β-actin (0.9 kb PstI fragment) all spotted on the same filters; all filters were split in half and hybridized with 2×10^6 cpm RNA per milliliter. The bar graph shows results (mean ± SD) from two experiments quantitated and normalized to β-actin values.

(C) Decay of *TNF*$^{\Delta ARE}$ (closed circles) and *TNF*$^{+}$ mRNAs (open circles) as assessed by the Northern analysis in (A). The results are expressed as percentage of density units from the peak of mRNA accumulation. Half-lives were calculated from the regression (dotted) lines of individual data sets.

(D) Detection of *TNF* pre- and mature mRNA in macrophages. Semiquantitative RT–PCR analysis on total RNA obtained from *TNF*$^{+/-}$ (left) and *TNF*$^{\Delta ARE/-}$ exudate peritoneal macrophages before (0) and at various time points after LPS stimulation. Results shown as Southern blots from PCR products run on 2% agarose gel and hybridized with cloned mTNF (0.9 kb NarI–BglII) and mGAPDH (0.17 kb AccI) fragments.

(E) Kinetics of TNF protein production per hour. Supernatants of *TNF*$^{\Delta ARE/-}$ (closed bars) and *TNF*$^{+/-}$ (hatched bars) exudate peritoneal macrophage cultures (5×10^5 adherent cells/ml medium) were collected at the hourly intervals before and after LPS stimulation, as before. Following each sample collection, cells were washed and incubated with fresh LPS-containing medium. TNF protein levels were assessed by ELISA. Data shown from a representative of three experiments from macrophages obtained from individual mice (n = 8). The time points shown correspond to the examined steady-state mRNA points examined earlier.

(F) Graphical correlation between the accumulation of *TNF*$^{\Delta ARE}$ (open circles) and *TNF*$^{+}$ (closed circles) mRNAs (dotted lines) and corresponding protein production (continuous lines) in the presence of LPS. Results shown as percentages of *TNF*$^{+}$ maxima.

dose of 10 μM Dex, protein production from *TNF*$^{\Delta ARE}$ mRNA could not be reduced to more than 50% of control levels, indicating a defect in the translational repression. Similar results were also obtained in BMDM from *TNF*$^{+/-}$ and *TNF*$^{\Delta ARE/-}$ mice as well as from macrophages derived from *TNF*$^{\Delta ARE/\Delta ARE}$/*TNFRI*$^{-/-}$ mice (data not shown). Taken together, these results establish the ARE as a specific target for the LPS-induced translational activation of TNF biosynthesis by the p38/SAPK and JNK/SAPK modules.

Notably, correlation of the levels of TNF protein produced per hour to the levels of the corresponding *TNF*$^{\Delta ARE}$ and *TNF*$^{+}$ mRNAs did not show significant differences (Figures 2E and 2F), suggesting that the absence of ARE does not influence the rate of translation of the mutant mRNA following LPS stimulation. It is therefore evident that TNF ARE regulate the translational activation but not the translational efficiency of the *TNF* message.

Absence of ARE Renders Nonhemopoietic Tissues Permissive for TNF Production

Earlier studies have favored the hypothesis that the ARE imposes a permanent dominant translational repression in nonhemopoietic cells (Kruys et al., 1993). We have therefore analyzed TNF biosynthesis in isolated primary fibroblast cultures derived from the synovium and lung of *TNF*$^{\Delta ARE/-}$ and *TNF*$^{+/-}$ mice for spontaneous or LPS-stimulated TNF expression. Using RT–PCR, the presence of *TNF*$^{+}$ mRNA was observed in cultured fibroblasts from both lung and synovium. A higher level of

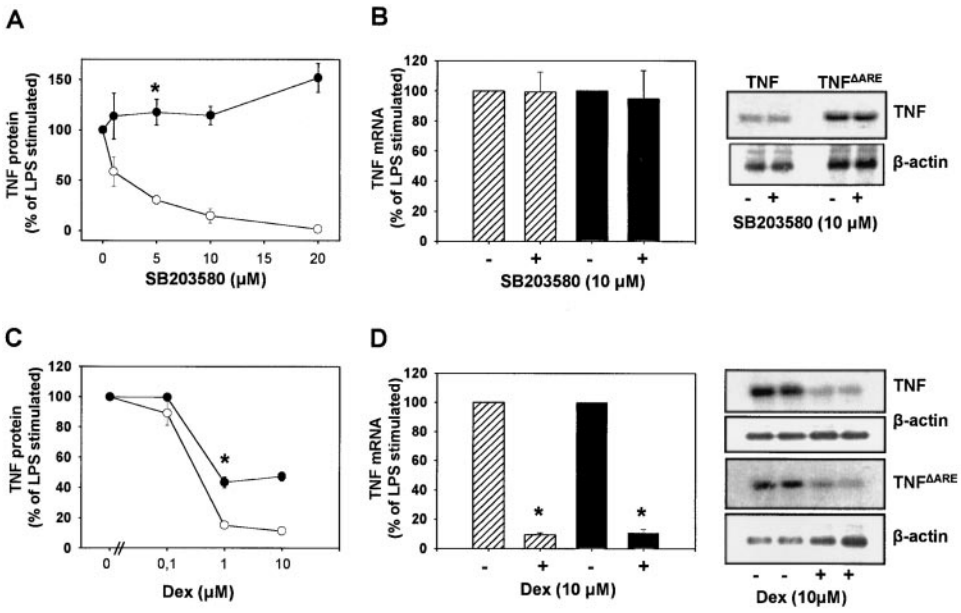

Figure 3. Effect of p38 and JNK/SAPK Inhibition on LPS-Induced TNF Production by Macrophages

$TNF^{\Delta ARE/-}$ (closed circles) and $TNF^{+/-}$ (open circles) TEPM's (5×10^5 adherent cells/ml) were stimulated with LPS (1 μg/ml) for 12 hr in the presence of various concentrations of the p38/SAPK inhibitor SB203580 (A) or dexamethasone (Dex) (C); TNF levels in supernatants were determined by ELISA. Northern analysis and quantitation of total RNA isolated from pooled (n = 4) $TNF^{\Delta ARE/-}$ (closed bars) and $TNF^{+/-}$ (hatched bars) macrophage cultures in the absence or presence of 10 μM SB203580 (B) or 10 μM dexamethasone (D). Results shown as percentages of LPS alone; values from three experiments with macrophages derived from individual mice (n = 8 mice/group/experiment).

$TNF^{\Delta ARE}$ mRNA could be recorded in either culture, indicating enhanced mRNA accumulation in the absence of the ARE (Figure 4A). Spontaneous TNF protein secretion could readily be detected in both synovial and lung $TNF^{\Delta ARE/-}$ and $TNF^{\Delta ARE/\Delta ARE}/TNFRI^{-/-}$ fibroblast cultures (Figure 4A). LPS stimulation of $TNF^{\Delta ARE/-}$ fibroblast cultures resulted in a further 2-fold increase in the levels of *TNF* mRNA and a 3-fold increase in TNF protein levels compared to unstimulated controls. In contrast, $TNF^{+/-}$ fibroblasts did not show any detectable changes in the levels of TNF mRNA or protein following LPS treatment.

Due to the very low levels of *TNF* mRNA in the $TNF^{+/-}$ fibroblasts, it has remained unclear whether the inability of this specific cell type to produce TNF protein results predominantly from translational repression or from the absence of a significant RNA pool. We have thus isolated synovial fibroblast-like cell cultures from a transgenic mouse line carrying a high copy number of a wild-type human *TNF* transgene (Keffer et al., 1991). In these transgenic mice, production of human TNF by TEPM parallels that of the endogenous murine TNF both in the absence or presence of LPS (Figure 4B). In contrast, despite the presence of a high level of human *TNF* mRNA in synovial fibroblast-like cells, no human TNF protein could be detected, indicating permanent suppression of translation. Taken together, these results suggest that in synovial or lung fibroblast-like cells (conceivably perhaps in other nonhemopoietic tissues as well), translation of wild-type *TNF* mRNA is silenced by the presence of the ARE region. Conversely, in the absence of the ARE, this translational control is no longer operative, resulting in the spontaneous production of TNF protein.

$TNF^{\Delta ARE}$ Mice Are Growth Retarded and Develop Chronic Inflammatory Arthritis and Inflammatory Bowel Disease

Homozygous $TNF^{\Delta ARE/\Delta ARE}$ mice show a severely reduced weight gain and increased mortality in comparison to heterozygous $TNF^{\Delta ARE/+}$ and normal littermate controls. Homozygous mice never exceeded 6–7 g of body weight and succumbed to disease between 5 and 12 weeks of age. In addition, these mice develop clinical evidence of chronic polyarthritis, presenting with swelling of several joints and distortion of front and rear paw morphology, which leads to a severe impairment in movement. Histological examination performed at different developmental stages in major organs of the $TNF^{\Delta ARE/\Delta ARE}$ and $TNF^{\Delta ARE/+}$ mice revealed the evolvement of two specific chronic pathologies, i.e., inflammatory arthritis and inflammatory bowel disease. Other organs from these mice such as liver and lung showed occasional evidence of mild inflammation, but tissue architecture was always well preserved. Thymi in 4-week-old $TNF^{\Delta ARE/\Delta ARE}$ mice were severely hypoplastic and showed atrophied and disorganized cortical and medullary areas. However, flow cytometric analysis of 12-day-old $TNF^{\Delta ARE/\Delta ARE}$ thymi and peripheral blood did not reveal any perturbation in the ratio of T cell subsets.

In agreement with macroscopic observations, the joints of $TNF^{\Delta ARE}$ mice displayed severe pathological features of chronic symmetrical inflammatory polyarthritis. The histological onset of the disease occurs earlier than 12 days postnatally in $TNF^{\Delta ARE/\Delta ARE}$ mice and at 6–8 weeks of age in $TNF^{\Delta ARE/-}$ mice. Early arthritic features include hyperplasia of the synovial membrane and the presence of polymorphonuclear infiltrates. At later stages, pannus

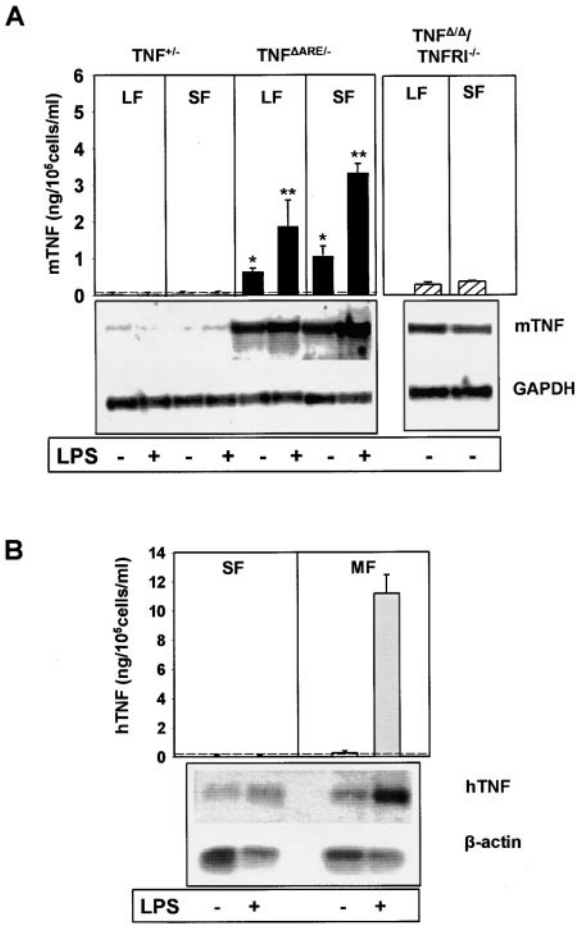

Figure 4. Spontaneous TNF Production by *TNF*$^{\Delta ARE}$ Lung and Synovial Fibroblasts

(A) *TNF*$^{+/-}$ (open bars), *TNF*$^{\Delta ARE/-}$ (closed bars), and *TNF*$^{\Delta ARE/\Delta ARE}$/*TNFRI*$^{-/-}$ (hatched bars) fibroblast cultures derived from lung (LF) and synovial (SF) tissue were examined by ELISA for the presence of TNF protein production in culture supernatants (10^5 adherent cells/ml) in the presence or absence of LPS (1 μg/ml) stimulation (pooled cultures of five animals per group). Values are mean (± SD) from at least three representative experiments. Detection of *TNF* mRNA via RT–PCR from total RNA derived from the above fibroblast cultures. Shown are Southern blots of the PCR products hybridized with muTNF- and GAPDH-specific probes. (B) Detection of hTNF protein and mRNA in synovial fibroblasts (SF) and TEMPs (MF) expressing an h*TNF* transgene in the presence or absence of LPS (1 μg/ml). Shown are ELISA values from three separate experiments and Northern analysis with an hTNF (0.6 kb EcoRI–NarI) genomic fragment; β-actin signals are shown for comparison.

and fibrous tissue formation, subchondral bone erosion, and articular cartilage destruction are observed (Figure 6A). All pathological characteristics were compatible with human rheumatoid arthritis, including the presence of rheumatoid factors (both IgM and IgG) (data not shown).

Systematic histological analysis of the gastrointestinal tract revealed profound inflammatory changes consistent with a Crohn's disease–like phenotype (Figure 5). These alterations were first detected in *TNF*$^{\Delta ARE}$ mice between 2 and 4 weeks of age and were localized primarily to the terminal ileum and occasionally to the proximal colon. The initial lesions consisted of mucosal abnormalities with intestinal villous blunting and broadening. These changes were associated with mucosal and submucosal infiltration of chronic as well as acute inflammatory cells, including mononuclear leukocytes, plasma cells, and scattered neutrophils. Severe intestinal inflammation was observed by 4 weeks in *TNF*$^{\Delta ARE/\Delta ARE}$ and 8 weeks in *TNF*$^{\Delta ARE/+}$ mice. An increased number of submucosal lymphoid aggregates and follicles were also evident at this time. As the disease progressed, the inflammatory infiltrate extended deep into the muscular layers of the bowel wall, with characteristics typical of transmural inflammation (Figure 5B). In aged *TNF*$^{\Delta ARE/+}$ mice (4–7 months old), complete loss of villous structures as well as rudimental granulomata resembling noncaseating granulomas with multinucleated giant cells were observed (Figure 5C). Overall, the histopathological findings in the intestines of *TNF*$^{\Delta ARE}$ mice closely resemble those observed in patients with Crohn's disease (Figure 5D).

Differential Role for TNFRII in the Modulation of Chronic Inflammatory Arthritis and Crohn's-like Pathology in *TNF*$^{\Delta ARE}$ Mice

To analyze the contribution of the two TNF receptors to disease development in the *TNF*$^{\Delta ARE}$ mutant mice, we have introduced the *TNF*$^{\Delta ARE}$ mutation into a TNFRI- or TNFRII-deficient genetic background (Rothe et al., 1993; Erickson et al., 1994). Double homozygous mice (*TNF*$^{\Delta ARE/\Delta ARE}$/*TNFRI*$^{-/-}$) develop normally, showing no apparent signs of macroscopic illness. Histopathological evaluation of mice at 1, 3, 6, and 8 months showed complete neutralization of all pathological complications, including intestinal and joint pathologies (Figures 5F and 6B). In contrast, *TNF*$^{\Delta ARE}$ mice bred into a TNFRII-deficient background did not show clinical improvement in comparison to *TNF*$^{\Delta ARE/+}$/*TNFRII*$^{+/-}$ controls. On the contrary, these mice showed a much more aggressive and destructive type of arthritis, which was clinically evident with enhanced swellings of the joints. At the histological level, double *TNF*$^{\Delta ARE/+}$/*TNFRII*$^{-/-}$ mice exhibited an exacerbated synovial hyperplasia with increased numbers of polymorphonuclear infiltrates and generally enhanced destruction of bone and cartilage in comparison to littermate controls (Figure 6C). These data indicate an overall suppressive role for TNFRII in the development of arthritic disease in this model. Notably, however, examination of *TNF*$^{\Delta ARE/+}$/*TNFRII*$^{-/-}$ intestinal tissues at 1–5 months of age revealed a marked attenuation of gut pathology, with only mild inflammatory changes localized to the mucosa and submucosa. No evidence of transmural inflammation, increased number of lymphoid aggregates, or granulomatous structures was detected compared to age-matched *TNF*$^{\Delta ARE/+}$/*TNFRII*$^{+/-}$ controls (Figure 5G). Taken together, these results demonstrate a dominant role for TNFRI in mediating the *TNF*$^{\Delta ARE}$ pathologies and reveal a differential role for TNFRII in modulating pathogenesis of chronic inflammatory arthritis and IBD in this model.

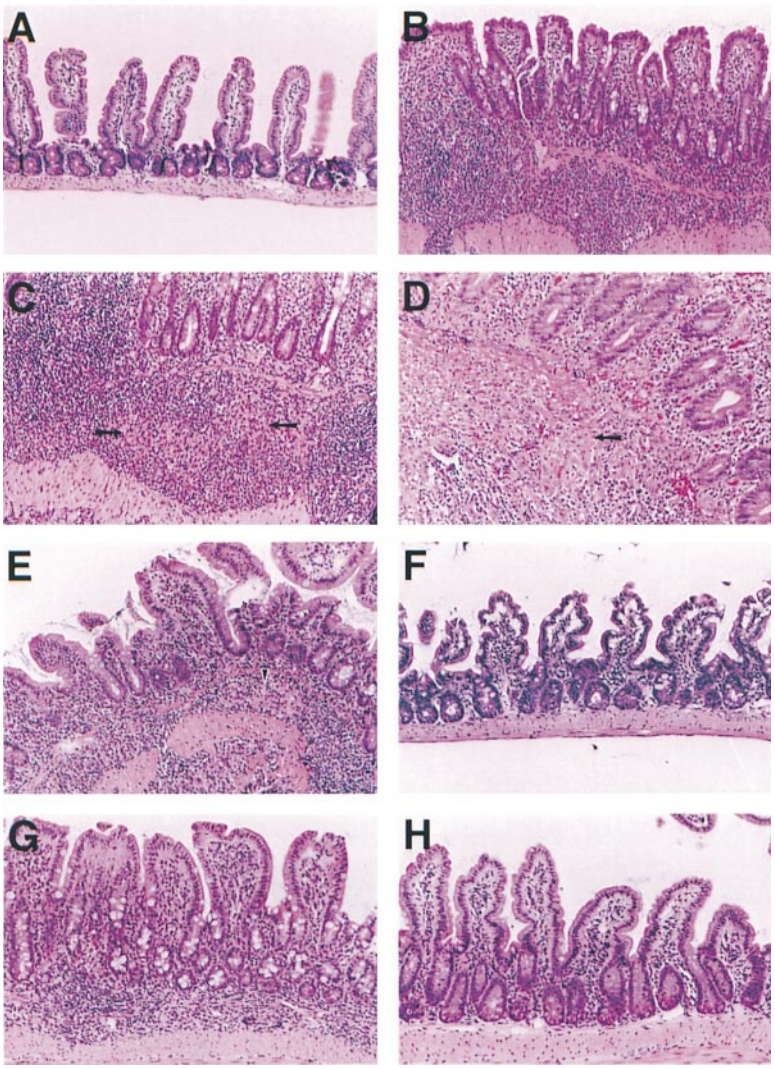

Figure 5. Inflammatory Bowel Disease in $TNF^{\Delta ARE}$ Mice Resembling Human Crohn's Disease

Histologic examination of portions of ileum from mice in different experimental groups.
(A) $TNF^{+/+}$ control at 8 weeks. The villus height and inflammatory component are normal.
(B) $TNF^{\Delta ARE/\Delta ARE}$ homozygote at 7 weeks. The changes are similar to those in the heterozygote 24 week animal. Villi are blunted and distorted, with a marked inflammatory infiltrate composed of lymphocytes, plasma cells, scattered neutrophils, and collections of submucosal histiocytes forming rudimentary granulomata.
(C) $TNF^{\Delta ARE/+}$ heterozygote at 16 weeks. There is villous blunting and chronic inflammation. An ill-defined noncaseating granuloma is present within the submucosa (arrowhead).
(D) Ileocecal region from a human with Crohn's disease. Note the similar location and composition of the granulomata (arrow) compared with the mouse model.
(E) $TNF^{\Delta ARE/+}$ heterozygote at 16 weeks. There is villus blunting with marked acute and chronic inflammation extending transmurally. An ill defined noncaseating granuloma is present within the submucosa (arrowhead).
(F) $TNF^{\Delta ARE/\Delta ARE}/TNFRI^{-/-}$ double homozygote at 12 weeks. There is normal intestinal appearance with preserved villi, minimal chronic inflammation confined to the mucosa, and unremarkable muscularis.
(G) $TNF^{\Delta ARE/+}/TNFRII^{-/-}$ at 16 weeks. There is a modest villous blunting with mild chronic inflammation and few neutrophils in the lamina propria without transmural inflammation.
(H) $TNF^{\Delta ARE/+}/RAG1^{-/-}$ at 12 weeks. There is mild patchy villous blunting and widening with a modest, almost normal chronic inflammatory infiltrate in the lamina propria only. There is no acute inflammation, nor is there acute inflammation in the muscularis mucosae or submucosa.
Hematoxylin and eosin staining. Magnification, ×150.

Differential Contribution of Mature T and B Cells in the Pathogenesis of TNF-Mediated Joint and Gut Pathology in $TNF^{\Delta ARE}$ Mice

Taking advantage of the simultaneous occurrence of two specific TNF-mediated pathologies in the same animal model, we wished to address the role of the adaptive immune response in this system. Introduction of the $TNF^{\Delta ARE}$ mutation into a *RAG1*-deficient background (Mombaerts et al., 1992) led to the generation of double mutant animals. *RAG1*-deficient $TNF^{\Delta ARE/+}$ mice displayed full clinical and histopathological signs of chronic destructive inflammatory arthritis (Figure 6D). In sharp contrast, the Crohn's-like intestinal phenotype was almost completely prevented in *RAG1*-deficient $TNF^{\Delta ARE/+}$ mice, with only minimal villous blunting and mild inflammatory changes limited to the intestinal mucosa (Figure 5H). Taken together, our findings demonstrate that in contrast to chronic inflammatory arthritis, the presence of mature T and B cells play a major role in the development of the IBD pathology in $TNF^{\Delta ARE}$ mice. Differential immune mechanisms are therefore responsible for the development of arthritis and IBD in the $TNF^{\Delta ARE}$ model.

Discussion

TNF ARE as Determinants of *TNF* mRNA Stability

ARE from early response genes (ERGs), proto-oncogenes, and cytokine mRNAs (Chen and Shyu, 1995) have been demonstrated to confer instability in chimeric reporter mRNAs. Previous studies, however, failed to show a role of the 3′-UTR/ARE on *TNF* message instability (Han et al., 1991b; Jacob et al., 1996). Our findings demonstrate that the absence of the ARE region results in increased steady-state *TNF* mRNA levels (Figures 2A and 3A) in both hemopoietic and stromal tissues, establishing *TNF* ARE as determinants of *TNF* mRNA instability in unstimulated conditions.

Determination of mRNA stability by ARE sequences in stimulated conditions appears complex and cannot be explained by static properties conferred by these

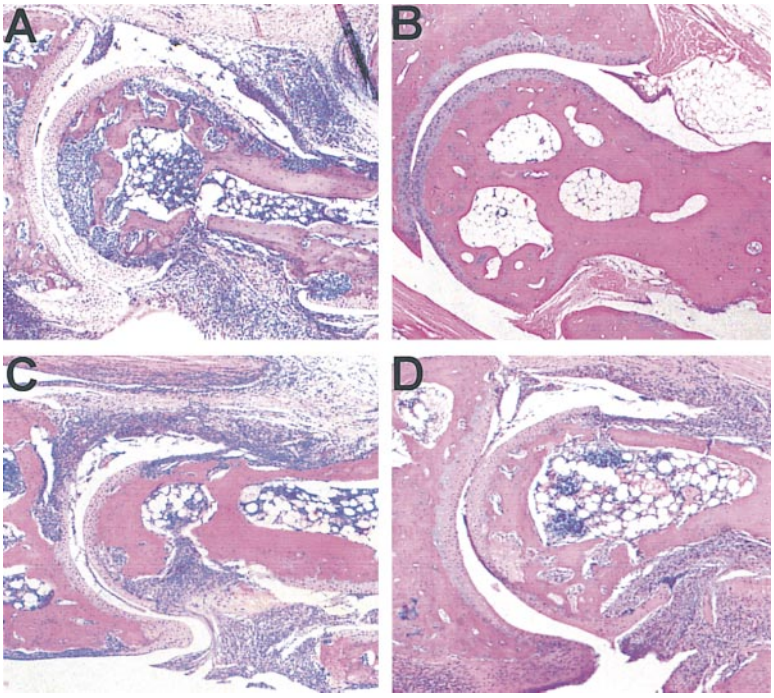

Figure 6. Chronic Inflammatory Polyarthritis in $TNF^{\Delta ARE}$ Mice

Histologic examination of ankle joints from mice in different experimental groups.
(A) $TNF^{\Delta ARE/\Delta ARE}$ homozygote at 7 weeks; the formation of the inflammatory pannus and areas of cartilage and bone erosion are evident.
(B) $TNF^{\Delta ARE/\Delta ARE}/TNFRI^{-/-}$ double homozygote at 7 months; no apparent signs of arthritis are observed.
(C) $TNF^{\Delta ARE/+}/TNFRII^{-/-}$ double mutant mice at 12 weeks; increased tissue destruction is evident, resembling the homozygote at 7 weeks.
(D) $TNF^{\Delta ARE/+}/RAG1^{-/-}$ double mutant at 12 weeks; arthritis similar to $TNF^{\Delta ARE/+}/RAG^{+/-}$ controls.
Hematoxylin and eosin staining. Magnification, ×40.

elements on the half-life of the mRNA, but rather by their active involvement in biphasic positive–negative regulatory loops. Initially, during the induction phase of gene expression, it appears that ARE mediate the stabilization of the respective mRNAs, leading to the accumulation of increased quantities of steady-state mRNA (Figures 2A and 2B). ARE-mediated stabilization should rely on a positively enforced mechanism, since fold stabilization of *TNF* mRNA is higher in the presence than in the absence of the ARE. It is noteworthy, however, that even in the absence of ARE, $TNF^{\Delta ARE}$ mRNA is still responsive to LPS-mediated stabilization. Therefore, it is possible that in addition to the ARE, other sequences mediate LPS-inducible and ARE-independent *TNF* mRNA stabilization. On the other hand, following a brief pulse of induction, destabilization mechanisms appear to be reinforced on the ARE, resulting in the rapid clearance of the wild-type message. Our data provide evidence that TNF ARE is involved in such negative feedback mechanisms, since, in its absence, $TNF^{\Delta ARE}$ mRNA shows a delayed onset of decay. Interestingly, induction of *TNF* mRNA destabilization has been demonstrated following thalidomide or PKC inhibitor administration in stimulated monocytes (Moreira et al., 1993) or astrocytes, respectively (Lieberman et al., 1990). It is therefore possible that these compounds necessitate the ARE to exert their function. Furthermore, tristetraprolin (TTP), a zing finger–containing protein, was recently shown to bind to AREs from several mRNAs, and its function was associated with negative regulation of the stability of the TNF message (Carballo et al., 1998). RNA stabilization by another family of proteins that show ARE binding activity, the HuR/ELAV family of proteins, has also been demonstrated recently (Peng et al., 1998), providing an example of an inducible factor operating on the ARE probably during the induction/stabilization phase of the response. These data support the concept that ARE sequences play a critical role in allowing the interplay of both positive and negative factors to determine the ordered functioning of stabilization and destabilization phenomena to occur.

TNF ARE as Determinants of *TNF* mRNA Translation

One of the most important mechanisms regulating TNF production that seems fully operative in quiescent macrophages is translational inhibition, which was previously shown to involve sequences present in the 3′-UTR of *TNF* mRNA (Han et al., 1990, 1991a). Here, the profound capacity of $TNF^{\Delta ARE}$ macrophages and T cells to produce TNF indicate permissive translation and confirm that the ARE should act as repressive elements in these specific cell types. However, although it appears that translational repression is reversible following stimulation of hemopoietic cells by appropriate inducers, it seems to operate permanently in nonhemopoietic tissues. A previous report suggested that in the latter cell types, TNF production may be totally prohibited due to either transcriptional restraints such as methylation or extinction of induced signaling pathways (e.g., LPS), leading to irreversible translational silencing (Kruys et al., 1993). We were able to detect wild-type *TNF* mRNA in wild-type synovial and lung fibroblast cultures, demonstrating that transcription is not hampered in such cells. However, despite detectable levels of wild-type *TNF* mRNA, protein production remained undetectable, confirming the translational silencing hypothesis. In addition, irreversible translational silencing of wild-type *TNF* mRNA in LPS-treated synovial fibroblasts could be demonstrated by transgenic overproduction of a wild-type *TNF* mRNA, which could not be translated in these cells (Figure 4B). In the absence of the ARE, however, both synovial and lung fibroblasts readily expressed enhanced levels of *TNF* mRNA and significant quantities of TNF protein,

indicating permissive translation. Although not addressed directly, increased RNA accumulation in the fibroblast should result from delayed decay kinetics in the absence of the ARE, as demonstrated for the macrophage.

The present experiments confirm earlier studies showing that translational activation is a significant mechanism accounting for the rapid induction of TNF protein production following LPS administration (Beutler et al., 1986) and assign a pivotal role for SAPK signals in this phenomenon (Prichett et al., 1995; Swantek et al., 1997). Most importantly, our data provide direct evidence that, in at least the macrophage, the ARE serve as targets for this type of signals, which therefore appear to orchestrate the on/off properties of the translational repressive capacity of the ARE. Negative regulation of the SAPK pathways by a number of anti-inflammatory stimuli is therefore expected to target the ARE to mediate suppression of TNF translation, and it is conceivable that functional disturbances on the ARE machinery may render translation of the *TNF* message unresponsive to anti-inflammatory modulation.

TNF ARE Mutation as an Etiopathogenic Mechanism for IBD and Arthritis

The appearance of an organ-specific rather than systemic disease profile, i.e., mainly IBD and arthritis, indicates a tissue-specific pathogenic potential of the ARE mutation. The IBD phenotype present in $TNF^{\Delta ARE}$ mice histologically most closely resembles human Crohn's disease. TNF has been suggested to significantly contribute to intestinal damage in Crohn's disease by altering the integrity of epithelial and endothelial cell barriers, increasing the recruitment of inflammatory cells, and contributing to the formation of granulomas (reviewed by van Deventer, 1997). In addition, the role of TNF has been shown directly in several animal models of IBD in which either anti-TNF treatments or its genetic ablation results in the amelioration of mucosal inflammation (Powrie et al., 1994; Neurath et al., 1997). The essential role of TNF in the pathophysiology of Crohn's disease has been unequivocally confirmed by the results of clear clinical benefit seen in the use of a single dose of anti-TNF monoclonal antibody in patients with active, therapy-refractory Crohn's disease (Targan et al., 1997).

Several animal models of IBD resembling ulcerative colitis have been produced (Strober et al., 1998). A suggested mechanism to explain disease pathogenesis in these systems has been either the absence of a lymphocytic regulatory component or the expansion of a pathogenic lymphocytic compartment (Powrie, 1995). We provide evidence that Crohn's disease pathogenesis in the $TNF^{\Delta ARE}$ mice requires the adaptive immune response and is dependent on the presence of both p55 and p75TNF-R, the latter being a receptor expressed mainly on hemopoietic cells (Santee and Owen-Schaub, 1996). It may therefore be speculated that direct modulation of hemopoietic cells by TNF is important in the maintenance of immunological homeostasis in the gut. The role of anti-TNF cytokines such as TGFβ and IL-10 has been also implicated in IBD (Shull et al., 1992; Kuhn et al., 1993). Thus, functional disturbances on the ARE machinery may render translation of the TNF message unresponsive to anti-inflammatory modulation, and failure to regulate this important homeostatic mechanism in the gut may result in the Crohn's-like phenotype characteristic of the $TNF^{\Delta ARE}$ model.

On the other hand, arthritis in $TNF^{\Delta ARE}$ mice develops even in the absence of a mature lymphocytic component or even in the absence of significant gut pathology (e.g., in the $RAG^{-/-}$ background). Our results suggest that the arthritic events occurring in the joints of the $TNF^{\Delta ARE}$ mice are driven by the spontaneous capacity of synoviocytes to produce TNF due to the absence of ARE (Figure 3). Similarly to the $TNF^{\Delta ARE}$ mice, synovial fibroblasts from arthritic *hTNF 3′-globin* transgenic mice also express high levels of TNF, and development of disease in a RAG1-deficient background remains unaltered (Douni et al., 1995). These data suggest parallel mechanisms of arthritis development in the two models and point toward the involvement of innate and/or stromal mechanisms in disease initiation and progression.

To our knowledge, no polymorphisms in the 3′-UTR of the *TNF* gene have so far been reported for humans. Given the regulatory complexity of this region, mutations affecting the ARE function may involve other associated factors. To that extent, it is interesting that the phenotype of mice lacking tristetraprolin (TTP) (Taylor et al., 1996), a factor which binds several ARE and appears to also regulate TNF biosynthesis (Carballo et al., 1998), shows partial resemblance to the $TNF^{\Delta ARE}$ model (i.e., arthritis but not IBD). In addition to such genetically defined mutations, exogenous factors such as viral proteins showing modulatory activity on ARE (Brown et al., 1995) may also affect TNF biosynthesis and be causative of disease.

Conclusions

Results reported in the present study clarify the regulatory properties of TNF ARE in several biosynthetic checkpoints and reveal the tissue-specific pathogenic potential of ARE mutations in whole animals. Our findings demonstrate that (1) TNF ARE impose a destabilizing activity on the *TNF* message in unstimulated conditions; (2) they contribute significantly to the permanent (nonreversible) translational silencing of the *TNF* message in nonhemopoietic tissues but also in the transient suppression of TNF translation in hemopoietic tissues; (3) following activation of hemopoietic cells by appropriate stimuli, ARE regulate positively both stabilization and translational derepression of the *TNF* message; (4) ARE serve as specific targets for translational activation by JNK/SAPK and p38MAPK-mediated signals; and (5) ARE are required for the restoration of homeostasis in TNF biosynthesis by mediating reinforcement of message destabilization and translational silencing following the induction phase of the response. Absence of this regulatory area from the mouse genome has led to a profound temporal and spatial deregulation of TNF production characterized by the persistent accumulation and decreased rates of decay of the mutant *TNF* mRNA and by its unresponsiveness to translational inhibition in hemopoietic and stromal tissues. Remarkably, this type of deregulation resulted in the development of chronic inflammatory arthritis and inflammatory bowel disease, suggesting a possible link between a defective

function of the ARE regulatory machinery and the development of analogous pathologies in humans. Our finding of a differential role of the two TNF receptors and of acquired immune mechanisms in the development of the joint and gut pathology in the *TNF*$^{\Delta ARE}$ mice establish this model as a unique tool for further comparative studies on the pathophysiology of these two diseases.

Experimental Procedures

Animals

All mice were bred and maintained on a mixed 129SvxC57BL/6 (targeted mutants) or CBAxC57BL/6 (transgenics) genetic backgrounds and kept in the animal facilities of the Hellenic Pasteur Institute under specific pathogen-free conditions.

Targeted Deletion of TNF ARE Elements

The *TNF* locus consisting of the *TNF*, *LT*α, and *LT*β genes was isolated from a 129/SvE mouse genomic library in λFix^{tm}II vector (Stratagene). A 0.7 kb fragment containing most of the 3′-UTR of the *TNF* gene was subcloned by EcoRI digestion into a pBluescript (SK-) vector (Stratagene) and was used as a PCR template for a site-directed deletion of 69 bp that contained 6 reiterated repeats of the (T)TAAAT(AT) motif. The following mutagenesis primer was used for the deletion: 5′-GAGCCAGCCCCCCTCGGAAGGCCGGG GTG-3′. The final PCR product was digested with BglII to remove a 0.4 kb 3′-UTR/ΔARE fragment, which was inserted back into the TNF 3′-UTR cassette. For the *TNF*$^{\Delta ARE}$ targeting vector, a 1.2 kb XhoI–SalI fragment from the *pL2-neo* plasmid containing the *PMC-neo-poly(A)* neomycin resistance cassette flanked by loxP sequences (Gu et al., 1994) was inserted in the BstEII site located in the 3′-UTR genomic sequence downstream of the ΔARE mutation. 5′ and 3′ homology regions of 4.2 kb (KpnI–EcoRI TNF/LTα fragment) and 1.4 kb (EcoRI–NdeI 3′TNF fragment) were inserted on either site of the modified TNF 3′-UTR cassette. The final *TNF*$^{\Delta AREneo}$ targeting fragment was retrieved as a KpnI–NotI 6.5 kb fragment and was subcloned into a *pNT* plasmid (Tybulewicz et al., 1991) bearing a herpes simplex virus thymidine kinase expression cassette. Targeting procedures in CCE (129/SvE mouse strain) embryonic stem cells and subsequent injections in C57BL/6 blastocysts were performed as previously described (Pasparakis and Kollias, 1995). Targeted clones were identified using Southern blot analysis with an external AccI 0.69 kb probe (Figures 1A and 1E). Transient *Cre* expression in the oocytes via microinjection of plasmid pMC-Cre-polyA (Araki et al., 1995) resulted in germline excision of the *neo* gene, as assessed by genotyping of tail biopsies from the resulting mice using a 0.4 kb BglII probe (Figures 1A, 1D, and 1F).

LPS-Induced Endotoxemia

Mice (10–12 wks of age) were injected intraperitoneally with the indicated amount of LPS (*Salmonella enteriditis*; Sigma L-6011) in sterile PBS per 25 g of mouse body weight. Mortality was monitored through a period of 7 days. For measurement of serum TNF, sera samples were collected via tail vein exsanguination before or 90 min after intraperitoneal administration of 100 μg LPS.

Cell Isolation and Culture

All cultures were grown at 37°C, 5% CO_2. Total exudate peritoneal macrophages (TEPM) were isolated by peritoneal lavage from 10-week-old old mice 3 days after a single intraperitoneal injection of 1.0 ml 4% thioglycollate broth (Difco Laboratories). The derivation of bone marrow–derived macrophages (BMDM) was performed as previously described by Warren and Vogel (1985). For all macrophage experiments, cells were plated at 5×10^5 cells/well in 24-well tissue culture plates in 1 ml of complete DMEM medium in the presence or absence of the indicated quantities of LPS (in PBS), dexamethasone (in ethanol) (Sigma), and the p38/SAPK inhibitor SB203580 (in DMSO) (donated by SmithKline Beecham).

For the fibroblast cultures, 10- to 12-week-old mice were sacrificed and perfused through the left cardiac ventricle with ice-cold PBS to remove circulating blood. Synovial and lung cultures were established by enzymatic treatment as previously described for human synovial tissue (Brennan et al., 1989). Fibroblasts were selected by continuous culturing for at least 20 days and a minimum of 3 passages. The uniformity of the cultures was assessed morphologically and by flow cytometry analysis for absence of myeloid (Mac-1) and lymphoid (CD3, B220) surface markers. For the experiments, fibroblasts were plated at 5×10^5 cells/well in 24-well tissue culture plates in 1 ml of complete DMEM for the times indicated.

TNF Protein Quantitation

Murine and human TNF immunoreactivity of sera (diluted 1/20–1/50) and cultured supernatants (diluted 1/4 −1/10) was measured using specific ELISAs as previously described (Pasparakis et al., 1996b). The sensitivity of these assays was at the range of 0.040–10 ng/ml.

RNA Analysis and Nuclear Run-On Assays

Total cellular RNA was extracted with the method of guanidinium isothiocyanate (Chomczynski and Sacchi, 1987). For Northern blot, a 0.9 kb ^{32}P-labeled NarI–BglII genomic probe containing part of the first exon of the mu*TNF* gene was used. This fragment is absent from the *TNF*$^{-/-}$ alleles (Pasparakis et al., 1996b).

For RT–PCR analysis, the following combinations of primers were used for the detection of the corresponding mRNA's: m*TNF*, sense 5′-CACGCTCTTCTGTCTACTGAACTTCG-3′ and antisense 5′-GGC TGGGTAGAGAATGGATGAACACC-3′; Pre-m*TNF* amplifying a fragment in the first exon/intron junction, 5′-AACCAGGCGGTTCTGT CCC-3′ and 5′-CTCTTGCTTATCCCCTCTTCC-3′; h*TNF*, 5′-TGCTGC ACTTTGGAGTGATCGG-3′ and 5′-GGTTATCTCTCQAGCTCCAC GCC-3′; and m*GAPDH*, sense 5′-TCTTCTTGTGCAGTGCC-3′ and antisense 5′-ACTCCACGACATACTCAGC-3′. PCR reactions were performed for 25 cycles to avoid product saturation. Products were subsequently diluted 1/20, resolved in 2% agarose gel, and blotted onto nylon membranes. Specific products were detected following hybridization with specific ^{32}P-labeled probes.

Isolation of macrophage nuclei and nuclear run-on reactions were performed as previously described (Linial et al., 1985). Slot blots were prepared containing DNA fragments corresponding to m*TNF* cDNA (1.6 kb) and β-actin (0.9 kb PstI fragment) as well as m*GAPDH* (0.17 kb AccI fragment) and pBluescript carrier plasmid control (data not shown), all spotted on the same filters (Hybond N+, Amersham). All filters were split in half and hybridized with an equal amount of labeled RNA (2×10^6 cpm/ml). Hybridization was performed for 48 hr in hybridization solution (50% formamide, 5× SSC, 5× Denhardt's solution, 0.02 M $NaHPO_4$ [pH 6.5], 100 μg/ml heat-denatured sheared salmon sperm DNA, and 10% dextran sulfate) at 42°C with 1×10^6 cpm/ml [^{32}P]RNA. Blots were washed as described with an RNAse step and autoradiographed for at least 48 hr at −70°C.

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